

Vitamin E Reduces Progression of Atherosclerosis in Low-Density Lipoprotein Receptor-Deficient Mice With Established Vascular Lesions

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Background—A growing body of evidence from animal studies supports the hypothesis that oxidative stress-mediated mechanisms play a central role in early atherogenesis. In contrast, clinical trials with antioxidant vitamins have not produced consistent results in humans with established atherosclerosis.

Methods and Results—Low-density lipoprotein receptor-deficient mice (LDLR KO) were fed a high-fat diet for 3 months to induce atheroma. At this time, 1 group of mice was euthanized for examination of atherosclerosis, and 2 other groups were randomized to receive high-fat diet either alone or supplemented with vitamin E for 3 additional months. At the end of the study, LDLR KO on a vitamin E-supplemented fat diet had decreased 8,12-iso-isoprostane (iP)F₂-VI and monocyte chemoattractant protein-1 levels, but increased nitric oxide levels compared with mice on placebo. No difference in lipid levels was observed between the 2 groups. Compared with baseline, placebo group had progression of atherosclerosis. In contrast, vitamin E-treated animals showed a significant reduction in progression of atherosclerosis.

Conclusions—These results demonstrate that in LDLR KO, vitamin E supplementation reduces progression of established atherosclerosis by suppressing oxidative and inflammatory reactions and increasing nitric oxide levels. (*Circulation*. 2003;107:521-523.)

Key Words: atherosclerosis ■ antioxidants ■ lipids ■ inflammation ■ nitric oxide

Atherosclerosis is a chronic disease influenced by multiple genetic and environmental factors that involves a complex interplay between blood components and the artery wall and is characterized by oxidative and inflammatory reactions.¹ Consistent data indicate that oxidative processes are of functional importance in animal models of atherosclerosis.² Epidemiological studies support these findings, indicating an inverse relationship between antioxidant vitamin intake and cardiovascular disease.³ Several clinical trials, however, have shown conflicting results as to whether or not antioxidant vitamins reduce atherosclerosis progression and cardiovascular events.⁴ Furthermore, in healthy subjects, vitamin E supplementation did not reduce the progression of the carotid artery intima-media thickness over a 3-year period.⁵

Several considerations can be made to explain these conflicting results, among them the endogenous antioxidant status of the study participants before enrollment and the timing of the intervention relative to the atherosclerotic process. It is plausible that in mice, vitamin E is effective because it is typically given at an early stage of the disease. In contrast, in humans, it has little or no effect because it is

administered when atherosclerotic lesions are already established.

Most animal studies have focused their attention on the effect of antioxidants on the formation of fatty streaks, the earliest cellular lesion of atherosclerosis. In contrast, the possible contribution of oxidative stress to the progression of atherosclerosis has been poorly investigated. Previously, we have shown that vitamin E suppresses *in vivo* lipid peroxidation and induces a significant reduction of early atherogenesis in different mouse models of atherosclerosis.^{6,7} The present study was designed to test the hypothesis that chronic attenuation of oxidative stress by vitamin E would have an impact on established atherosclerosis in low-density lipoprotein receptor deficient (LDLR KO) mice.

Methods

Animal Experimental Protocol

All procedures were approved by the Institutional Committee. LDLR KO mice (n=42, Jackson Laboratories, Bar Harbor, Me) were allowed to age to 12 months and were then fed a high-fat diet (normal chow supplemented with 0.15% cholesterol and 20% butter fat) for a total of 12 weeks prior baseline analysis. At this time-point blood was drawn, plasma cholesterol was quantitated, and mice were

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divided in 3 groups of 14 animals each, with mean cholesterol levels that were not significantly different. One group of animals was killed immediately for analysis of atherosclerosis (baseline group). The remaining mice were randomized to receive high-fat diet alone or supplemented with vitamin E (2.1 U/g diet) for 3 additional months. Blood samples were obtained from mice at baseline and then at monthly intervals until the end of the study.

Biochemical Analyses

Plasma cholesterol and triglyceride levels were measured enzymatically and vitamin E levels were assayed by high-performance liquid chromatography as previously described.^{4,7} Total plasma NO metabolites were evaluated with measurements of nitrite+nitrate by a colorimetric assay (Assay Design). Plasma 8,12-*iso*-iPF₂-VI levels were measured by gas chromatography/mass spectrometry, as previously described.^{7,9} Levels of soluble intercellular adhesion molecules-1 (s-ICAM1) and monocyte chemoattractant protein-1 (MCP-1) were measured by ELISA kits (Endogen, Inc and R&D System).^{7,9}

Preparation of Mouse Aortas and Quantitation of Atherosclerosis

After the final blood collection, mice were euthanized and the aortic tree was perfused for 10 minutes with ice-cold PBS containing 20 μ M/L BHT and 2 mmol/L EDTA (pH 7.4) as previously described.^{7,9} After removal of the surrounding adventitial fat tissue, the aorta was opened longitudinally, fixed in formal-sucrose, and stained with Sudan IV. The extent of atherosclerosis was determined using the en face method, in a blinded fashion as previously described.^{4,7}

Histology and Immunohistochemistry

Briefly, serial frozen sections of the aortic root of the proximal aorta, starting at the sinus, were examined. Immunostaining for macrophage content was performed as previously described.^{7,9} Briefly, a Mab to mouse macrophages (MOMA-2; Accurate Chemicals) and a Mab anti-human smooth muscle α -actin (Sigma Chemical Co) for smooth muscle cells were used. Antibody reactivity was detected using a Nova red substrate kit (SK-4800, Vector Laboratory). Cross sections were counterstained with hematoxylin. As control, no primary antibody was added to the same sections. Images of immunostained sections were captured and analyzed in a blinded fashion as previously described.^{7,9}

Statistical Analysis

Results were expressed as mean \pm SEM. Data were analyzed by ANOVA and subsequently by Student's unpaired 2-tailed *t* test, as indicated. Probability values less than 0.05 were considered as significant.

Results

Vitamin E Effects on Plasma Lipids

Compared with baseline group, mice from the placebo group showed a further significant increase in both plasma cholesterol and triglycerides. This increase was also evident in LDLR KO mice receiving the high-fat diet supplemented with vitamin E (Table). No significant difference in lipid levels was found between mice on vitamin E or high-fat diet alone. Compliance with vitamin E dietary regimen was demonstrated by a significant increase in its circulating levels in mice on vitamin E-enriched diet (Table). Elevation of plasma vitamin E levels was also evident when the values were normalized for cholesterol (data not shown).

Vitamin E Effects on Oxidative and Inflammatory Processes

Plasma levels of 8,12-*iso*-iPF₂-VI, a major F₂-isoprostane and a specific marker of lipid peroxidation,⁸ were further

Characteristics of the 3 Groups of Mice

	Baseline	High-Fat Diet	
		Placebo	Vitamin E
Cholesterol, mg/dL	800 \pm 50	1150 \pm 100†	1115 \pm 85
Triglycerides, mg/dL	450 \pm 45	710 \pm 60†	680 \pm 70
Vitamin E, μ M	20 \pm 2	16 \pm 1.8†	52 \pm 2.2*
8,12- <i>iso</i> -iPF ₂ -VI, pg/mL	750 \pm 60	1100 \pm 55†	630 \pm 50
sICAM-1, ng/ml	11 \pm 1.5	14 \pm 2	10 \pm 2*
MCP-1, ng/ml	200 \pm 15	245 \pm 21	180 \pm 16*
Nox, μ M	30 \pm 3.2	18 \pm 2.6†	48 \pm 2.4*

Each group includes 14 mice. Results are expressed as mean \pm SEM.

**P*<0.05 vs placebo; †*P*<0.05 vs baseline.

elevated in LDLR KO mice kept on a high-fat diet alone when compared with the baseline group. In contrast, vitamin E significantly reduced these levels to values that were similar to the ones observed in mice at baseline (Table). At the end of the study, mice on the high-fat diet alone had a further increase in s-ICAM-1 and MCP-1 circulating levels, whereas vitamin E significantly reduced them (Table). Because impaired NO synthesis has been described in hypercholesterolemia,¹⁰ we examined the effect of vitamin E supplementation on NO metabolite (Nox) levels. Compared with baseline, plasma Nox levels were further reduced in mice on a high-fat diet alone. In contrast, vitamin E supplementation preserved higher plasma Nox levels compared with both baseline and the placebo group (Table).

Vitamin E Effects on Preexisting Atherosclerotic Lesions

The Sudan IV-stained aorta preparations of the LDLR KO mice on the high-fat diet for 3 months showed atherosclerotic lesions mainly localized in the sinus and arch portions, covering 11.2 \pm 1.4% of the entire vessel (Figure 1). The aortas from mice receiving a high-fat diet for 3 additional months demonstrated further progression of atherosclerosis, which involved the thoracic and abdominal portions of the aorta. In contrast, this area was significantly reduced in LDLR KO mice on high-fat diet supplemented with vitamin E (Figure 1). No significant difference was observed with the baseline group (Figure 1).

Immunohistochemical analyses of aortic root sections showed no difference in the percentage area occupied by

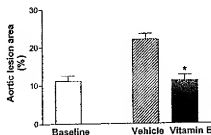


Figure 1. Percentage of total aortic atherosclerotic lesion areas in LDLR KO mice fed a high-fat diet for 3 months (baseline), and those fed a high-fat diet alone or with vitamin E for 12 additional weeks (*n*=14 per group). **P*<0.01 versus placebo.

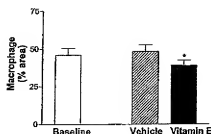


Figure 2. Percentage area of aortic root atherosclerotic lesions occupied by macrophages in LDLR KO mice fed high-fat diet for 3 months (baseline), and those fed a high-fat diet alone or with vitamin E for 12 additional weeks ($n=8$ per group). * $P<0.01$ versus placebo.

macrophages between baseline and placebo group after normalized to total lesion area. However, this area was significantly reduced in mice receiving vitamin E compared with placebo (Figure 2). Finally, no difference in smooth muscle cell content was observed among the 3 groups (data not shown).

Discussion

In the current study, we demonstrated for the first time that chronic supplementation of vitamin E retards the progression of established atherosclerotic lesions in LDLR KO mice on a high-fat diet by decreasing oxidative and inflammatory reactions and increasing NO levels.

Lipid peroxidation, in particular oxidative modification of LDL in vivo, is thought to play a functional role in atherogenesis.² Evidence consistent with this hypothesis includes the presence of oxidized lipids in atherosclerotic lesions and the reduction of murine atherosclerosis by structurally distinct antioxidants.^{11–13} Several trials have shown, however, that antioxidants do not reduce the risk of fatal or non-fatal infarction in an unselected population with established atherosclerosis.⁴ These conflicting results do not necessarily mean that the oxidative hypothesis of atherosclerosis is incorrect. It is possible that the animal intervention studies deal primarily with early lesions, whereas clinical trials deal with established ones. We have previously shown that in vivo lipid peroxidation is increased in the apolipoprotein E-deficient mice and LDLR KO mice, and that its inhibition by vitamin E coincides with a reduction in atherosclerosis.^{6,7} It is plausible that in humans antioxidants have little or no effect because they are given when lesions are already established. To test this hypothesis, LDLR KO mice initially kept on a high-fat diet for 3 months were subsequently randomized to either receive a high-fat diet supplemented with vitamin E or stay on the high-fat diet alone for 12 additional weeks. We found that vitamin E reduced progression of atherosclerosis without affecting lipid levels by suppressing oxidative stress. These results support the concept that vitamin E is effective in LDLR KO mice whether it is given at the early phase of atherogenesis or after the disease is established.

Atherosclerosis is associated with oxidative stress, which is characterized by a reduction of endogenous antioxidants and NO levels.^{14,15} We confirmed these data by showing that vitamin E restores and increases these levels. Reactive oxygen species can interact with NO and produce peroxynitrate, which in turn can further sustain oxidative injury to the endothelium. By restoring the endogenous antioxidant status, vitamin E increases NO levels and limits peroxynitrate formation, which could then act as additional antiatherogenic mechanisms by reducing vascular inflammation. Indeed, our present findings demonstrate that by reducing oxidative stress, vitamin E improves indices of inflammation and endothelial function, which are critically involved in the progression of atherosclerosis.

In interpreting our results, we must consider an important limitation of this study; no animal model mimics perfectly human atherosclerosis. Despite this fact, our study supports the hypothesis that the discrepancies between animal studies and clinical trials with vitamin E cannot be explained by the timing of the intervention, ie, early versus established lesions.

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References

- Glass CK, Witztum JL. Atherosclerosis: the road ahead. *Cell*. 2001;104:503–516.
- Witztum JL, Steinberg D. The oxidative modification hypothesis of atherosclerosis: does it hold for humans. *Trends Cardiovasc Med*. 2001;11:93–102.
- Rimm EB, Stampfer MJ, Ascherio A, et al. Vitamin E consumption and the risk of coronary heart disease in men. *N Engl J Med*. 1993;328:1450–1456.
- Pratico D. Vitamin E: murine studies versus clinical trials. *Ital Heart J*. 2001;2:878–881.
- Holts EB, Mack WJ, LaBree L, et al. Alpha-tocopherol supplementation in healthy individuals reduces low-density lipoprotein oxidation but not atherosclerosis. *Circulation*. 2002;106:1453–1459.
- Pratico D, Tangirala RK, Rader DJ, et al. Vitamin E suppresses isoprostane generation in vivo and reduces atherosclerosis in ApoE-deficient mice. *Nat Med*. 1998;4:1189–1192.
- Cyrus T, Tang LX, Kockach J, et al. Lipid peroxidation and platelet activation in murine atherosclerosis. *Circulation*. 2001;104:1940–1945.
- Pratico D, Lawson JA, Kockach J, et al. The isoprostanes in biology and medicine. *Trends Endocrin Metab*. 2001;12:243–247.
- Pratico D, Cyrus T, Zhang Z, et al. Acceleration of atherogenesis by COX-1-dependent prostanoic acid formation in low-density lipoprotein receptor deficient mice. *Proc Natl Acad Sci U S A*. 2001;98:3358–3363.
- Ishikawa I, Sugawara D, Wang X, et al. Heme oxygenase-1 inhibits atherosclerotic lesion formation in LDL-receptor knockout mice. *Circ Res*. 2001;88:506–512.
- Witztum JL, Berliner JA. Oxidized phospholipids and isoprostanes in atherosclerosis. *Curr Opin Lipidol*. 1998;9:441–448.
- Tangirala RK, Casanueva F, Miller E, et al. Effect of the anti-oxidant N,N'-diphenyl 1,4-phenylene diamine (DPPD) on atherosclerosis in apoE-deficient mice. *Arterioscler Thromb Vasc Biol*. 1994;15:1625–1630.
- Cynshi O, Kawabe Y, Suzuki T, et al. Antiatherogenic effects of the antioxidant BO-653 in three different animal models. *Proc Natl Acad Sci U S A*. 1998;95:10123–10128.
- Pratico D. Lipid peroxidation in mouse models of atherosclerosis. *Trends Cardiovasc Med*. 2001;11:112–116.
- Oemar B, Tschütdi MR, Godoy N, et al. Reduced endothelial synthase expression and production in human atherosclerosis. *Circulation*. 1998;97:2494–2498.